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Replication of phage ϕ29 DNA with purified terminal protein and DNA polymerase: Synthesis of full-length ϕ29 DNA

(protein-primed initiation/phage ϕ29 DNA polymerase/phage ϕ29 terminal protein)

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ABSTRACT **A system that replicates bacteriophage ϕ29 DNA with protein p3 covalently attached to the two 5′ ends, using as the only proteins the ϕ29 DNA polymerase and the terminal protein, is described. Restriction analysis of the ^{32}P-labeled DNA synthesized *in vitro* showed that all ϕ29 DNA fragments were labeled. Analysis by alkaline sucrose gradient centrifugation of the DNA labeled during a 10-min pulse showed that, after a 20-min chase, about half of the DNA molecules had reached apparently full-length ϕ29 DNA (≈18,000 nucleotides). Ammonium ions strongly stimulated ϕ29 DNA-protein p3 replication, the effect being due to stimulation of the initiation reaction. ATP was not required for ϕ29 DNA-protein p3 replication, either in the initiation or elongation steps. The results show that the ϕ29 DNA polymerase functions, not only in the formation of the p3-dAMP covalent initiation complex but also in the elongation of the latter, as the only DNA polymerase to produce full-length ϕ29 DNA.**

Bacteriophage ϕ29 from *Bacillus subtilis* has a linear, double-stranded DNA of about 18,000 base pairs (1), with protein p3, product of the viral gene 3, covalently linked to the two 5′ ends (2–5) through a phosphoester bond between the OH group of a serine residue and 5′ dAMP (6). The replication of ϕ29 DNA is initiated at either DNA end (7–9) by a mechanism of protein-priming in which a free molecule of the terminal protein p3, in the presence of ϕ29 DNA-protein p3 as template, reacts with dATP in a reaction that requires the product of the viral gene 2 (10, 11) and forms a protein p3-dAMP covalent complex that provides the 3′-OH group needed for elongation (12, 13). The product of gene 2 has been shown to be a phage ϕ29-encoded DNA polymerase (14, 15). When highly purified proteins p2 (DNA polymerase) and p3 (terminal protein) were used in the *in vitro* initiation reaction with ϕ29 DNA-protein p3 as template, formation of the p3-dAMP complex was greatly stimulated by extracts from uninfected *B. subtilis* or from *Escherichia coli*, suggesting the involvement of some host factor(s) in the initiation reaction (15).

The phage ϕ29 DNA polymerase functions not only in the initiation step of replication, but also it can catalyze ϕ29 DNA chain elongation at least up to nucleotides 9 and 12 from the left and right DNA ends, respectively, when a limited elongation assay was carried out (15, 16). In addition, the ϕ29 DNA polymerase has a 3′ → 5′ exonuclease activity on single-stranded DNA that probably acts as a proofreading mechanism (17, 18).

In this paper we describe a system that replicates phage ϕ29 DNA-protein p3 using as the only proteins the ϕ29 DNA polymerase and the terminal protein. The p3-dAMP covalent complex formed in the initiation step is elongated giving rise to apparently full-length ϕ29 DNA. The requirements for this replication system are also described. The rate of elongation with this two-protein minimal system is low, suggesting that other viral and/or cellular accessory proteins are used *in vivo* for ϕ29 DNA replication.

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MATERIALS AND METHODS

Assay for Formation of the p3-dAMP Initiation Complex. The incubation mixture contained, in 0.05 ml, 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM spermidine, 0.25 μM [α-^{32}P]dATP (5 μCi; 1 Ci = 37 GBq), 1 μg of ϕ29 DNA-protein p3, 300 ng of purified protein p3 (19) obtained from I. Prieto, 20 ng of purified protein p2 (15) and, when indicated, 3 μg of a fraction of uninfected *B. subtilis* cells prepared as described by Blanco and Salas (15) as a source of host factor(s). After incubation for 20 min at 30°C, the samples were filtered through Sephadex G-50 spun-columns (20) and processed as described by Peñalva and Salas (12).

Replication Assay With Phage ϕ29 DNA-Protein p3 as Template. The incubation mixture was as described for the initiation reaction except that it contained 10 μM [α-^{32}P]dATP (2 μCi); 20 μM each dGTP, dCTP, and dTTP (P-L Biochemicals), 1 mM ATP; 5% (vol/vol) glycerol; and bovine serum albumin (0.1 mg/ml). Besides purified proteins p2 and p3, the components indicated in each case were added. After 20 min at 30°C, the reaction was stopped by adding 10 mM EDTA/0.1% $NaDodSO_4$ and heating for 10 min at 68°C, and the samples were filtered through Sephadex G-50 spun-columns as described (20). The excluded fraction was counted by Cerenkov radiation.

Elongation Assay with Phage ϕ29 DNA-Protein p3 as Template. Purified proteins p2 and p3 were incubated with phage ϕ29 DNA-protein p3 complex as template for 10 or 15 min at 30°C in the presence of [α-^{32}P]dATP as indicated in each case. Then, anti-p3 IgG (24 μg) was added to stop further initiations and, at the indicated times, dATP up to 10 μM was added as well as 20 μM of dCTP, dTTP, and dGTP. The incubation was continued at 30°C with the additions indicated in each case. At different times, samples were removed, the reaction was stopped by addition of 10 mM EDTA/0.1% $NaDodSO_4$ and heating for 10 min at 68°C, and the labeled DNA was subjected to restriction analysis as described below.

Restriction Analysis of the Replicated ϕ29 DNA. The DNA labeled in the replication and elongation assays described above with phage ϕ29 DNA-protein p3 as template was treated with proteinase K (200 μg/ml) in the presence of 0.5% $NaDodSO_4$ during 5 hr at 37°C. The samples were filtered through Sephadex G-50 spun-columns, and the excluded fractions were treated with phenol/chloroform as described (20), precipitated with ethanol, and treated with *Hin*dIII. The fragments were separated in a 3.5% polyacrylamide slab gel. After electrophoresis, the gel was dried and autoradiographed with intensifying screens at −70°C. Quantitation was

done by excising the bands from the gel and counting the Cerenkov radiation.

Alkaline Sucrose Gradient Analysis of the Replicated Phage ϕ29 DNA. The DNA labeled in the replication assay described above during 10 min at 30°C with 10 μM [α-^{32}P]dATP (5 μCi) and ϕ29 DNA-protein p3 as template was chased for 10 min and then for 20 additional min at 30°C in the presence of 0.5 mM dATP. Samples were taken after the pulse and after each time of chase, denatured by treatment with 0.1 M NaOH, and subjected to centrifugation for 2.5 hr at 189,000 $\times$ *g* in a 5–20% (wt/vol) sucrose gradient in 0.1 M NaOH/0.9 M NaCl/10 mM EDTA. Proteinase K-treated ϕ29 DNA, prepared as described by Inciarte *et al.* (21), was added to each sample as an internal marker because it was shown that the presence of the terminal protein in ϕ29 DNA did not affect its sedimentation position. Fractions were taken from the bottom of the tubes, and the absorbancy at 260 nm and the Cerenkov radiation were determined.

RESULTS

Elongation of the p3-dAMP Initiation Complex by the Phage ϕ29-Specific DNA Polymerase. It was previously shown that purified phage ϕ29-induced DNA polymerase (protein p2), in addition to catalyzing the formation of the p3-dAMP initiation complex, was able to elongate the ϕ29 DNA chain, at least up to nucleotides 9 and 12 from the left and right DNA ends, respectively (15). To determine whether further elongation could be carried out by the purified ϕ29 DNA polymerase, labeled DNA synthesized by using the ϕ29 DNA-protein p3 complex as template in the presence of highly purified proteins p2 and p3 was digested with *Hin*dIII, and the fragments were subjected to polyacrylamide gel electrophoresis. All of the *Hin*dIII restriction fragments were labeled (Fig. 1*A*, lane a). When the ratio of radioactivity to DNA length of each fragment was plotted as a function of their relative order in the ϕ29 genome, it was seen that the terminal fragments were labeled more efficiently than the internal ones (Fig. 1*B*), as expected from the fact that new initiations were taking place at both DNA ends during the time of incubation. Very low incorporation took place when protein p3 was not added (Fig. 1*A*, lane b), indicating that most of the labeling was due to specific elongation from the p3-dAMP initiation complex and it was not due to a repair reaction.

We have shown (15) that some factor(s) from the host cell greatly stimulates the formation of the p3-dAMP initiation complex when added to purified proteins p2 and p3 with ϕ29 DNA-protein p3 as template. The host factor(s) was completely inactivated by treatment with proteinase K, indicating its protein nature (unpublished results). To determine the effect of the host factor(s) on elongation, an experiment similar to that described above was carried out. When host factor(s) was added to purified proteins p2 and p3, there was an increase in the labeling of the terminal fragments, but the internal ones were labeled to an extent similar to that obtained in the absence of host factor(s) (Fig. 1*A*, lane c, and Fig. 1*B*). As a control, when protein p3 was not added, very low incorporation took place (Fig. 1*A*, lane d), again indicating that the labeling was due to elongation from the p3-dAMP initiation complex.

Synthesis of Full-Length Phage ϕ29 DNA. To determine whether full-length phage ϕ29 DNA was being synthesized and whether the appearance of label in all of the restriction fragments was due to full elongation from each end, a pulse–chase experiment was carried out. The DNA labeled with [α-^{32}P]dATP during 10 min in the presence of the ϕ29 DNA polymerase (p2) and the terminal protein (p3) with ϕ29 DNA-protein p3 complex as template was chased with an excess of cold dATP for different times, and the labeled DNA was analyzed by alkaline sucrose gradient centrifugation. The DNA labeled in the 10-min pulse had a mean size of 2.2 $\times$ 10^6 daltons (37% of the DNA length) (Fig. 2). After a chase of 10 min, the mean size increased to 3.7 $\times$ 10^6 daltons (63% of the DNA length), and after 20 min of chase, about half of the DNA molecules reached the size corresponding to full-length ϕ29 DNA (5.9 $\times$ 10^6 daltons).

Requirements for the Replication of Phage ϕ29 DNA-Protein p3 with Purified ϕ29 DNA Polymerase and Terminal Protein. The terminal protein p3, ϕ29 DNA polymerase p2, ϕ29 DNA-protein p3 complex, and Mg^{2+} are essential for replication activity (Table 1). Proteinase K-treated ϕ29 DNA could not replace the ϕ29 DNA-protein p3 complex as template. Removal of ATP slightly stimulated the replication activity (see below). Addition of $(NH_4)_2SO_4$ greatly stimulated ϕ29 DNA-protein p3 replication, the optimal concentration being around 20 mM. Removal of protein p3 in the presence of 20 mM $(NH_4)_2SO_4$ essentially abolished ϕ29 DNA-protein p3 replication. The effect of other salts was also

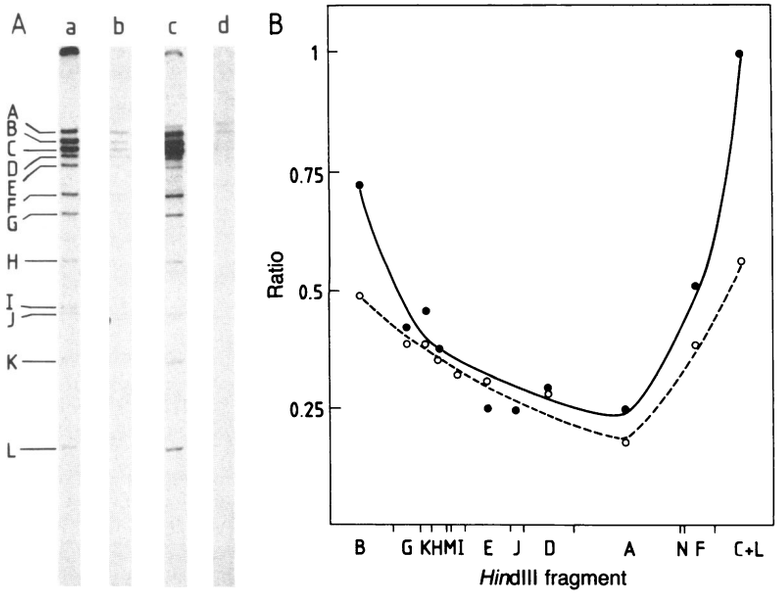

FIG. 1. Replication of phage ϕ29 DNA-protein p3 with purified ϕ29 DNA polymerase (p2) and terminal protein (p3) and effect of host factor(s). (*A*) DNA labeled with 10 μM [α-^{32}P]dATP (5 μCi) after 20 min at 30°C in the replication assay with ϕ29 DNA-protein p3 complex (0.5 μg) as template and purified proteins p2 (10 ng) and p3 (64 ng) was treated with proteinase K and *Hin*dIII and subjected to polyacrylamide gel electrophoresis as described. After electrophoresis, the gel was dried and autoradiographed. Lanes: a, complete system (1.16 pmol of dNMP incorporated); b, without p3 (0.28 pmol of dNMP incorporated); c, with host factor(s) (3 μg) (2.04 pmol of dNMP incorporated); d, without p3 and with host factor(s) (3 μg) (0.2 pmol of dNMP incorporated). (*B*) Each band from the gel in *A*, lanes a and c, was cut out, and the radioactivity was determined. The ratio of radioactivity to DNA length of each fragment was plotted, normalizing to 1.0 the value for the right terminal fragments C + L in the presence of host factor(s). The actual incorporation in the C + L fragments was 0.16 pmol of dNMP with proteins p2 and p3 (---) and 0.28 pmol with proteins p2, p3, and host factor(s) (—).

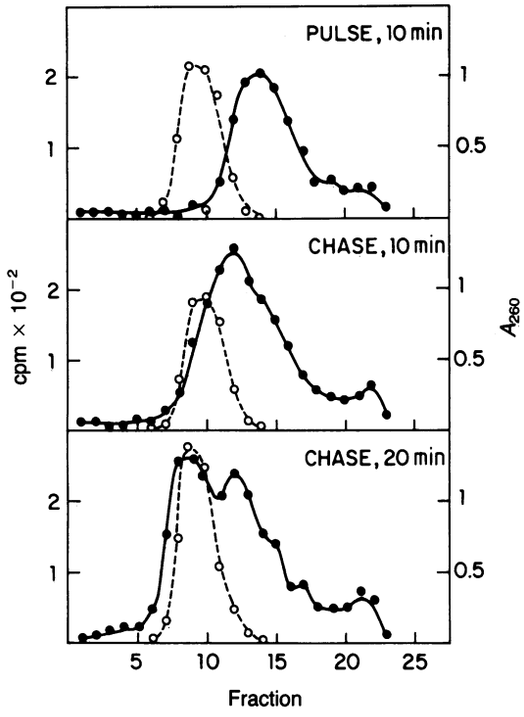

FIG. 2. Synthesis of full-length ϕ29 DNA with purified phage ϕ29 DNA polymerase (p2) and terminal protein (p3). The DNA labeled with 10 μM [α-^{32}P]dATP (5 μCi) during a 10-min pulse in the replication assay with ϕ29 DNA-protein p3 complex (1 μg) as template and purified proteins p2 (20 ng) and p3 (128 ng) was chased for 10 min and for 20 additional min, denatured, and subjected to alkaline sucrose gradient centrifugation as described in the presence of proteinase K-treated ϕ29 DNA (30 μg) as marker. —, ^{32}P-labeled DNA; ---, marker ϕ29 DNA.

tested. NH_4Cl had an effect similar to that of $(NH_4)_2SO_4$, KCl also stimulated, although to a lower extent, and NaCl had no effect (Table 1).

$(NH_4)_2SO_4$ Stimulates the Formation of the p3-dAMP Initiation Complex. The stimulation by $(NH_4)_2SO_4$ was further analyzed by studying the effect of this salt on the initiation reaction. $(NH_4)_2SO_4$ stimulated the initiation reaction when added to purified proteins p2 and p3, the optimal concentration being around 20 mM (Fig. 3, lanes a–e). As already reported (15) and shown in lane f of Fig. 3, addition of host factor(s) from uninfected *B. subtilis* stimulated the initiation reaction. Addition of 20 mM $(NH_4)_2SO_4$ to the reaction containing host factor(s) produced a further stimulation (Fig. 3, lane g). Bovine serum albumin up to 20 μg had no effect whether in the absence or presence of 20 mM $(NH_4)_2SO_4$ (Fig. 3, lanes h and i). Fig. 4*A* shows the kinetics in the formation of the p3-dAMP initiation complex in the absence or presence of 20 mM $(NH_4)_2SO_4$. A similar effect of $(NH_4)_2SO_4$ was obtained when replication of ϕ29 DNA was analyzed (Fig. 4*B*), suggesting that the $(NH_4)_2SO_4$ is mainly affecting the initiation reaction in the replication of ϕ29 DNA-protein p3. No significant effect of $(NH_4)_2SO_4$ on the K_m value for dATP in the initiation reaction was observed.

To determine whether $(NH_4)_2SO_4$, in addition to its effect in initiation, affects the elongation reaction in phage ϕ29 DNA-protein p3 replication, initiation was allowed to occur for 15 min with 10 μM [α-^{32}P]dATP in the absence of $(NH_4)_2SO_4$. Then, anti-p3 IgG was added to stop most of the further initiations; after 5 min, the remaining dNTPs were added, and the incubation was continued for 7.5 min in the absence or presence of 20 mM $(NH_4)_2SO_4$. The samples were treated with *Hin*dIII, and the fragments were subjected to polyacrylamide gel electrophoresis. No effect of the $(NH_4)_2SO_4$ was observed on the relative labeling of the different restriction fragments, suggesting that $(NH_4)_2SO_4$ is not affecting the rate of elongation in ϕ29 DNA replication (results not shown). In agreement with this result is the fact that, when purified ϕ29 DNA polymerase was used with activated nicked DNA as template, only a small stimulation (a factor of 1.5) over the control was obtained in the presence of 20 mM $(NH_4)_2SO_4$.

Effect of ATP on Initiation and Elongation in Phage ϕ29 DNA-Protein p3 Replication. Removal of ATP slightly stimulated the replication of phage ϕ29 DNA-protein p3 (Table 1). The effect of ATP on initiation with purified proteins p2 and p3 is shown in Fig. 5. Addition of 1 mM ATP reduced to about

Table 1. Requirements for the replication of phage ϕ29 DNA-protein p3 with purified DNA polymerase p2 and terminal protein p3

System	Activity
Complete	1
− protein p3	0.018
− protein p2	0.01
− ϕ29 DNA-protein p3	0.002
− ϕ29 DNA-protein p3, + proteinase K-ϕ29 DNA	0.001
− Mg^{2+}	0
− ATP	1.3
+ 10 mM $(NH_4)_2SO_4$	6.6
+ 20 mM $(NH_4)_2SO_4$	13.4
+ 30 mM $(NH_4)_2SO_4$	12.9
+ 60 mM $(NH_4)_2SO_4$	3.9
+ 20 mM $(NH_4)_2SO_4$ − protein p3	0.02
+ 20 mM NH_4Cl	10.2
+ 40 mM NH_4Cl	12.3
+ 20 mM KCl	7.2
+ 40 mM KCl	7.9
+ 20 mM NaCl	0.9
+ 40 mM NaCl	0.8

The replication assay was carried out for 20 min at 30°C as described. The value of relative activity 1 represents the incorporation of 8.4 pmol of dNMP.

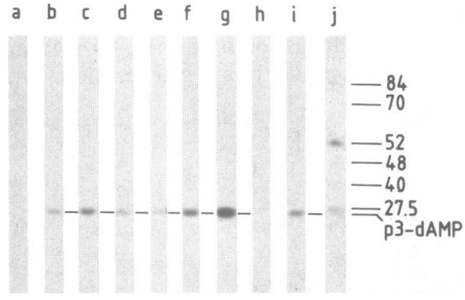

FIG. 3. Effect of $(NH_4)_2SO_4$ on the formation of the protein p3-dAMP initiation complex. Purified proteins p2 (20 ng) and p3 (300 ng) were incubated for 20 min at 30°C with phage ϕ29 DNA-protein p3 complex (1 μg) as template in the presence of 0.25 μM [α-^{32}P]dATP (5 μCi) with the additions indicated in each case and were assayed for the formation of the p3-dAMP initiation complex as described. Lanes: a, no addition; b, with 10 mM $(NH_4)_2SO_4$; c, with 20 mM $(NH_4)_2SO_4$; d, with 30 mM $(NH_4)_2SO_4$; e, with 60 mM $(NH_4)_2SO_4$; f, with 3 μg of host factor(s) from uninfected *B. subtilis*; g, as in lane f plus 20 mM $(NH_4)_2SO_4$; h, with 20 μg of bovine serum albumin; i, as in lane h plus 20 mM $(NH_4)_2SO_4$; j, ^{35}S-labeled ϕ29 structural proteins. Quantitation of the p3-dAMP complex formed was done by excising the band from the gel and counting the Cerenkov radiation. The amount of p3-dAMP complex formed was, in fmol, <0.1 (lane a), 0.28 (lane b), 0.56 (lane c), 0.25 (lane d), 0.18 (lane e), 0.59 (lane f), 1.88 (lane g), <0.1 (lane h), and 0.48 (lane i). Size is shown in daltons × 10^{-3}.

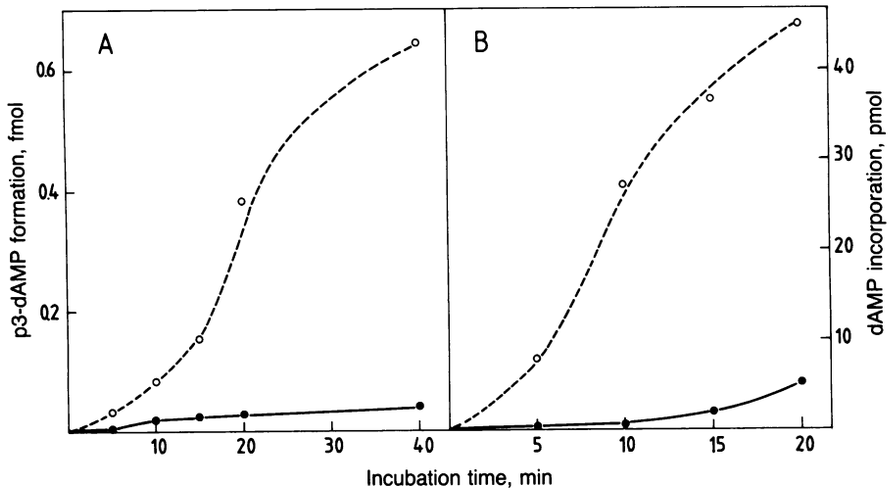

FIG. 4. Effect of $(NH_4)_2SO_4$ on the time kinetics in the initiation and replication assays with purified phage ϕ29 DNA polymerase (p2) and terminal protein (p3) and ϕ29 DNA-protein p3 as template. (*A*) Purified proteins p2 (20 ng) and p3 (300 ng) were incubated for the indicated times at 30°C with ϕ29 DNA-protein p3 complex (1 μg) as template in the presence of 0.25 μM [α-^{32}P]dATP (5 μCi) for the formation of the p3-dAMP initiation complex as described in the absence (—) or presence (---) of 20 mM $(NH_4)_2SO_4$. The radioactivity present in the p3-dAMP complex was quantitated by excising the band from the gel and counting the Cerenkov radiation. (*B*) Purified proteins p2 (20 ng) and p3 (300 ng) were incubated for the indicated times at 30°C with ϕ29 DNA-protein p3 complex (1 μg) as template in the presence of 10 μM [α-^{32}P]dATP (2 μCi) in the replication assay as described in the absence (—) or presence (---) of 20 mM $(NH_4)_2SO_4$.

40% the formation of the p3-dAMP initiation complex when 0.25 μM dATP was used (Fig. 5*A*, lanes a–c). However, when the concentration of dATP was increased to 10 μM, addition of 1 mM ATP had no effect on the initiation reaction (Fig. 5*B*, lanes a and b), suggesting that the ATP inhibition is due to competition with the dATP. To find out whether ATP is required for the elongation reaction in ϕ29 DNA-protein p3 replication, initiation was allowed to occur for 10 min in the absence of ATP. Then, dNTPs were added as well as anti-p3 IgG to stop further initiations, and the incubation was continued for 10 min to allow elongation in the absence or presence of different ATP concentrations. The labeled DNA was digested with *Hin*dIII and subjected to polyacrylamide gel electrophoresis. Fig. 6 shows that all of the restriction fragments were similarly labeled in the absence of ATP (lane a) or in the presence of 0.1, 0.3, or 1 mM ATP (lanes b–d), indicating that ATP has no effect on the rate of elongation.

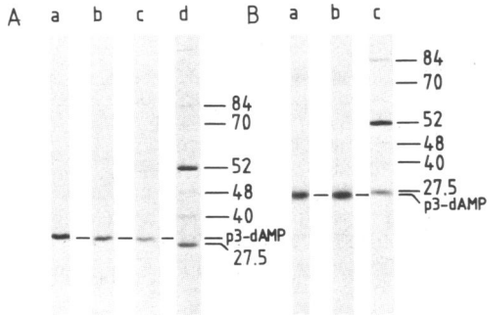

FIG. 5. Effect of ATP on the formation of the protein p3-dAMP initiation complex. (*A*) Purified proteins p2 (20 ng) and p3 (300 ng) were incubated for 20 min at 30°C in the presence of 20 mM $(NH_4)_2SO_4$ with phage ϕ29 DNA-protein p3 complex (1 μg) as template and 0.25 μM [α-^{32}P]dATP (5 μCi) in the absence of ATP (lane a) or in the presence of 0.1 mM (lane b) or 1 mM (lane c) ATP, and the formation of the p3-dAMP complex was determined as described. ^{35}S-labeled ϕ29 structural proteins are in lane d. (*B*) As in *A*, except that 10 μM [α-^{32}P]dATP (10 μCi) was added in the absence (lane a) or presence (lane b) of 1 mM ATP. ^{35}S-labeled ϕ29 structural proteins are in lane c.

Quantitation of the radioactivity present in each band confirmed the above conclusion.

DISCUSSION

The purified phage ϕ29 DNA polymerase, in the presence of the terminal protein and ϕ29 DNA-protein p3 complex as template, had previously been shown to function in the formation of the p3-dAMP initiation complex and in the elongation of this complex up to nucleotides 9 and 12 from the

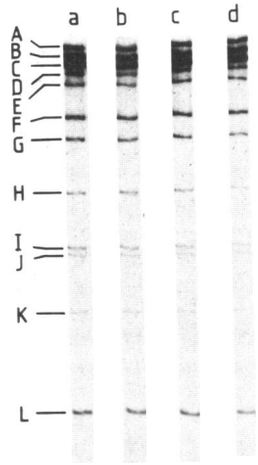

FIG. 6. Effect of ATP on the elongation in phage ϕ29 DNA-protein p3 replication. Purified proteins p2 (20 ng) and p3 (300 ng) were incubated for 10 min at 30°C in the presence of 20 mM $(NH_4)_2SO_4$ with ϕ29 DNA-protein p3 complex (1 μg) as template in the presence of 0.25 μM [α-^{32}P]dATP (5 μCi) and in the absence of ATP. Then, anti-p3 IgG (24 μg) was added to stop further initiations as well as 10 μM dATP and 20 μM dCTP, dTTP, and dGTP, and the reaction was continued for 10 min in the absence (lane a) or presence of 0.1 mM (lane b), 0.3 mM (lane c), or 1 mM (lane d) ATP. The samples were treated with proteinase K and *Hin*dIII and subjected to polyacrylamide gel electrophoresis as described. After electrophoresis, the gel was dried and autoradiographed. Quantitation was done by excising the bands from the gel and counting the Cerenkov radiation.

left and right ϕ29 DNA ends, respectively, when a limited elongation assay was carried out, suggesting that protein p2 has a role in the elongation step in ϕ29 DNA-protein p3 replication (15, 16, 22). We now show that, in fact, the ϕ29 DNA polymerase is able to synthesize apparently full-length ϕ29 DNA molecules. It is interesting to stress the fact that only two viral-encoded proteins, the DNA polymerase and the terminal protein, can replicate the ϕ29 DNA-protein p3 template *in vitro*; the p3-dAMP complex formed in the initiation step can be elongated, giving rise to full-length ϕ29 DNA. The host factor(s), shown to stimulate the ϕ29 initiation reaction (15), essentially did not affect the extent of elongation. The stimulation observed in the labeling of the terminal fragments could be an indirect one due to the effect on initiation.

Adenovirus DNA replication, which also takes place by a protein-priming mechanism, requires three viral-encoded proteins (preterminal protein, DNA polymerase, and single-stranded DNA binding protein) and two host factors (nuclear factors I and II) to obtain the synthesis of full-length adenovirus DNA (23). In the absence of the nuclear factor II, shown to contain a topoisomerase I-like activity, the products obtained were only 25% of the size of full-length DNA (23). A possibility for the need of a topoisomerase I-like activity to obtain full-length DNA in the case of adenovirus replication and not in phage ϕ29 replication could be the fact that adenovirus DNA is about twice the size of ϕ29 DNA.

The results presented in this paper indicate that ATP is not required either for initiation or elongation in the replication of phage ϕ29 DNA-protein p3. It is possible that hydrolysis of dNTPs during polymerization can supply enough energy for replication. Previous experiments using crude or partially purified systems had shown stimulation by ATP in the formation of the p3-dAMP initiation complex (10–12, 24) or when ϕ29 DNA-protein p3 replication was analyzed (25). A possible explanation for this difference is that, when crude systems are used, ATP might protect against dNTP breakdown, as has been suggested in the case of adenovirus replication (26).

The replication of phage ϕ29 DNA-protein p3 was greatly stimulated by $(NH_4)_2SO_4$, the effect being due to an increase in the amount of p3-dAMP initiation complex formed. No effect of $(NH_4)_2SO_4$ on elongation was observed, suggesting that the stimulation is not on the DNA polymerase activity of p2. In addition, the $3' \rightarrow 5'$ exonuclease activity of p2 was not affected by $(NH_4)_2SO_4$ (unpublished results). The effect of $(NH_4)_2SO_4$ in the initiation *in vitro* could be a stimulation of the interaction between proteins p2 and p3 and/or of the interaction between the protein(s) and DNA.

From the data of the pulse–chase experiment (Fig. 2), a value of about 10 nucleotides per sec at 30°C can be calculated for the rate of elongation in phage ϕ29 DNA-protein p3 replication with purified p2 and p3 as the only proteins in the *in vitro* system. This value is similar to the one reported for adenovirus DNA replication with the five purified proteins indicated before (23). Although no data are available on the rate of ϕ29 DNA elongation *in vivo*, the rate of fork movement in *E. coli* is approximately 1000 nucleotides per sec (27). This suggests that, in addition to the ϕ29 DNA polymerase, other accessory proteins are likely to be involved in ϕ29 DNA replication *in vivo* for proper catalytic, processivity, fidelity, and other features of the physiological system.

Other viral proteins that are involved in phage ϕ29 DNA replication *in vivo* are the products of genes *5*, *6*, and *17* (28–30). The products of genes *5* and *6* were shown to be involved in elongation *in vivo* (31), and the fact that extracts from *sus17*-infected *B. subtilis* can form the p3-dAMP initiation complex *in vitro* (10, 11) might suggest that the gene *17* product is also involved in elongation. The gene *6* product has been recently purified and shown to stimulate the initiation reaction *in vitro* (32). Preliminary evidence suggests that p6 may be a single-stranded DNA binding protein since it binds to denatured DNA but not to native DNA. It remains to be determined whether p6, as well as p5, and p17 are involved in elongation processes in ϕ29 DNA replication.

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